Mitochondrial dysfunction is an early event in high-NaCl-induced apoptosis of mIMCD3 cells

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Received 25 September 2001; accepted in final form 6 December 2001

Michea, Luis, Christian Combs, Peter Andrews, Natalia Dmitrieva, and Maurice B. Burg. Mitochondrial dysfunction is an early event in high-NaCl-induced apoptosis of mIMCD3 cells. Am J Physiol Renal Physiol 282: F981–F990, 2002. First published January 29, 2002; 10.1152/ajprenal.00301.2001.—Raising osmolality to 700 mosmol/kgH2O by the addition of NaCl rapidly kills most murine inner renal medullary collecting duct cells (mIMCD3), but they survive at 500 mosmol/kgH2O. At 300 and 500 mosmol/kgH2O, NADH autofluorescence is present in a mitochondria-associated, punctate perinuclear pattern. Within 45 s to 30 min at 700 mosmol/kgH2O, the autofluorescence spreads diffusely throughout the cell. This correlates with mitochondrial membrane depolarization, measured as decreased tetramethylrhodamine methyl ester perchlorate (TMRM) fluorescence. Mitochondrial dysfunction should increase the cellular ADP/ATP ratio. In agreement, this ratio increases within 1–6 h. Mitochondrial morphology (transmission electron microscopy) is unaffected, but nuclear hypercondensation becomes evident. Progressive apoptosis occurs beginning 1 h after osmolality is raised to 700, but not to 500 mosmol/kgH2O. General caspase activity and caspase-9 activity increase only after 6 h at 700 mosmol/kgH2O. The mitochondrial Bcl-2/Bax ratio decreases within 1–3 h, but no cytochrome c release is evident. The mitochondria contain little p53 at any osmolality. Adding urea to 700 mosmol/kgH2O does not change NADH or TMRM fluorescence. We conclude that extreme acute hypertonicity causes a mitochondrial dysfunction involved in the initiation of apoptosis.

caspase; endonuclease G; cytochrome c; mitochondria membrane potential; murine inner medullary collecting duct; sodium chloride

During the production of concentrated urine, cells of the renal medulla experience osmotic concentrations of NaCl in the interstitium exceeding 1,000 mosmol/kgH2O (8), which they survive. In contrast, renal medullary cells in tissue culture die rapidly by apoptosis after acute addition of NaCl that raises the osmolality above 600 mosmol/kgH2O (35, 45). Mitochondria are known to play a pivotal role in triggering and coordinating apoptosis (24), making this organelle a logical candidate to evaluate in NaCl-induced apoptosis in mIMCD3 cells.

Hypertonicity, induced by high NaCl, decreases cell volume, increases cytosolic osmolality, and changes mitochondrial osmotic equilibrium, which could affect mitochondrial function. Mitochondria normally have a high negative membrane potential that provides a driving force for entry of cations from the cytoplasm. Resulting net uptake of cations by mitochondria, followed by osmotic swelling, constitutes a threat to their osmotic integrity. Mitochondrial volume is normally maintained by the kinetic equilibrium between electrophoretic K+ entry and electroneutral K+ efflux via the K+/H+ antiporter (10, 23). The influence of matrix volume on mitochondrial metabolism has been studied in isolated mitochondria in vitro. Experiments designed to alter matrix volume demonstrate strong effects on mitochondrial metabolism (2, 26, 40). Acute hypertonic stress inhibits substrate oxidation, reduces respiration, and decreases ATPase activity (3, 17, 18, 26, 34, 37, 40). The time course of these changes depends on the nature and concentration of the osmolyte used to increase toxicity, and the changes are reversible below 500 mosmol/kgH2O (10, 17, 18). These observations could be relevant to the mechanism of hypertonicity-induced apoptosis. Mitochondria can initiate apoptosis when, for example, drugs or chemical agents depolarize their membranes, uncouple respiration, and inhibit ATP synthesis.

Mitochondrial membrane potential is a good indicator of their energy status, reflecting proton electrochemical gradient across the inner membrane. A decrease in membrane potential is associated with the release of apoptogenic factors from the mitochondria, and in several models of apoptosis is the first index of mitochondrial dysfunction (16). Members of the Bcl-2 family of proteins participate in the changes of the mitochondrial membrane permeability involved in the collapse of membrane potential and the release of apoptogenic factors. Bcl-2 is normally present in mito-
Mitochondrial dysfunction and functions as a repressor of apoptosis (43, 53). Bax, a proapoptotic Bcl-2 family member, is a soluble protein predominantly present in the cytosol (11, 21, 25, 29). During the induction of apoptosis, Bax shifts to membranes, including those in mitochondria. Furthermore, Bax and Bcl-2 mutually antagonize each other when coexpressed in the same cell, and the ratio of Bcl-2/Bax in mitochondria determines the cellular response to death signals transmitted by mitochondria (16, 39, 50). Inhibition of apoptosis by Bcl-2 may result from homodimerization or oligomerization with Bax, maintenance of mitochondrial homeostasis resulting in prevention of apoptogenic factors release, and/or inhibition of the production of reactive oxygen species (1, 25, 54, 55).

In view of these considerations, we hypothesized that acute hypertonic stress in mIMCD3 cells might critically affect mitochondrial function. Thus the resultant apoptosis could be the consequence of mitochondrial dysfunction, possibly involving changes in the Bcl-2/Bax ratio and the release of apoptogenic signaling molecules from mitochondria. We tested our hypothesis by measuring mitochondrial function and apoptosis in mIMCD3 cells stressed by addition of NaCl to the medium. A combination of biochemical and morphological methods was used to characterize changes in mitochondrial structure and the localization of cytochrome c, Bax, Bel-2, and p53.

METHODS

Cell culture. Murine inner renal medullary collecting duct cells (mIMCD3), generously provided by S. Gullans, were cultured on 100-mm Falcon plastic dishes and used between passages 16 and 20. The cells were harvested by trypsinization in Ca²⁺/Mg²⁺-free Dulbecco’s phosphate-buffered saline (DPBS) and seeded at the densities indicated below to be used at 70–80% confluence in the experiments. Cells were fed with 1:1 Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Irvine) containing 10% fetal calf serum and 2 mM-glutamine at 37°C in 5% CO₂. The osmolality of the basal medium was 300 mosmol/kgH₂O (Advanced Osmometer, Norwood, MA).

Mitochondrial NADH and membrane potential. All fluorescence measurements were made using a Zeiss LSM 510 confocal microscope and a ×63 C-Apo (1.2 numerical aperture) water-correction objective. The pinhole was adjusted to produce an optical slice thickness as specified (see figure legends). For experiments localizing NADH autofluorescence and estimating changes in mitochondrial membrane potential with tetramethylrhodamine methyl ester perchlorate (TMRE; 20 nM), cells (1,000,000) were grown on sterile cover glasses (no. 1, 22 × 40 mm, Warner Instrument, Hamden, CT) and then immersed in a Falcon dish (100 mm) for 48 h. The cover glass was mounted in a microscope perfusion chamber (Warner Instrument), and NADH autofluorescence was measured (351-nm excitation, LP 385-nm emission). The cells were bathed with 300 mosmol/kgH₂O medium (37°C, 5% CO₂) for 20 min for equilibration, and the medium was then exchanged quickly for the experimental hypertonic medium (500 or 700 mosmol/kgH₂O final osmolality) during the indicated periods. The mitochondrial origin of signals was confirmed by colocalization with 50 nM Mitotracker green (mitochondrial-specific dye, Molecular Probes, Eugene, OR, 488-nm excitation, LP 505-nm emission) and the disappearance of the fluorescent signal with 100 nM FCCP (mitochondrial uncoupler). For mitochondrial membrane potential experiments, cells were loaded with 20 nM TMRE (543-nm excitation, LP 585-nm emission) for 20 min at 37°C in a humidified incubator (5% CO₂), mounted in the perfusion chamber, and bathed as described above.

Cell fixation and propidium iodide staining. To measure cell survival and nuclear condensation and for immunofluorescence experiments, mIMCD3 cells were cultured on 8-chamber plastic slides (Permanox, Nalgé Nunc), seeding 7,500 cells/chamber, and studied 48 h later. After the experimental intervention, the cells were fixed in 100% methanol at −20°C for 20 min, permeabilized with 0.1% Triton X-100, incubated with 1 mg/ml RNase (Sigma) for 15 min, stained with 10 μg/ml propidium iodide (PI) for 5 min, and then mounted with 200 μl of SlowFade antifade solution (Molecular Probes, Eugene, OR).

Laser scanning cytometry. Cell number and apoptosis were determined with a laser scanning cytometer (LSC; CompuCyte, Cambridge, MA) by measuring total and peak intensity of PI fluorescence in each nucleus, as previously described (9, 19, 22). Total nuclear PI fluorescence is the integral of fluorescence calculated over the entire area of a nucleus. It corresponds to DNA content and is used for cell-cycle analysis (WinCyte software, CompuCyte). The peak intensity is the highest pixel value within the nucleus. This increases as nuclear chromatin condenses during apoptosis. The data are displayed as bivariate cytograms, plotting peak PI fluorescence vs. total PI fluorescence in each nucleus or particle. A gate representing the approximate limit of peak fluorescence in control cells under isotonic conditions was set by eye. The validity of the gate was confirmed by microscopic examination of cells inside and outside the putative apoptotic region of the cytograms. These cells were located on the slide by using the spatial coordinates recorded for each cell by the LSC, and the cells were visualized with the charge-coupled device camera of the LSC in the epifluorescence mode. Once the gate was validated, it was utilized to analyze the cytograms obtained from cells on the same slide in different experimental conditions. Nuclei with high peak fluorescence appeared bright and shrunken, consistent with the hypercondensation of chromatin that occurs early in apoptosis. Finally, the number of viable cell nuclei was calculated as the number of single nuclei with normal peak fluorescence and a DNA content between 2 and 4 N.

Immunofluorescence. To visualize cytochrome c and cytochrome oxidase complex IV (COX IV), the cells were fixed using 3.7% paraformaldehyde in DPBS (GIBCO Life Sciences) for 15 min at room temperature. Cells were washed four times (3 min each) with 500 μl of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100 (TBST) at room temperature and blocked with 5% bovine serum albumin (fraction V, Sigma) in TBST for 1 h. For cytochrome c immunofluorescence, the cells were incubated with affinity-purified anti-cytochrome c mouse IgG1 (Pharmlingen, Becton-Dickinson), at an IgG concentration of 0.10 mg/ml, in 3% bovine serum albumin-TBST for 1 h. After being washed with TBST (3 times, 5 min each), the cells were incubated with 1:200 dilution of goat anti-mouse IgG antibody linked to Alexa 488 (Molecular Probes) and counterstained with PI (1 μg/ml, Sigma) in DPBS containing RNase A (0.5 mg/ml, Sigma). For COX IV immunodetection, after the washing steps, the cells were incubated with mouse monoclonal IgG2a at a concentration of 10 μg/ml (Molecular Probes) in TBST for 1 h, washed three times with TBST (5 min each), and incu-
bated for 1 h with 1:200 dilution of goat anti-mouse, highly cross-adsorbed IgG antibody linked to Alexa 488 (Molecular Probes). Then, the cells were washed twice with 500 μl of TBST (3 min each), and nuclei were counterstained with PI (1 mg/ml, Sigma) in DPBS containing RNase A (0.5 mg/ml, Sigma). For p53 immunolocalization, cells were incubated with mouse anti-p53 IgG<sub>2A</sub> (1:200 dilution, Roche Molecular Biochemicals) in TBST for 1 h and detected with goat anti-mouse highly cross-adsorbed IgG antibody linked to Alexa 488 (Molecular Probes). The slides were mounted in SlowFade (Pernaxon slides) antifade solution (Molecular Probes) for examination using the epifluorescence microscope (BX50, Olympus, Melville, NY) of the LSC (Compucyte). Digital images were acquired with a Kodak Digital Science DC 120 zoom digital camera.

Subcellular fractionation and preparation of mitochondrial fractions. For mitochondrial isolation, 3,500,000 mIMCD3 cells grown on 150-mm culture dishes (Corning, Corning, NY) in isotonic medium were fractionated using an ApoAlert Cell Fractionation Kit (Clontech). After 48 h, the cells were incubated for 1 or 3 h with prewarmed fresh medium. The cells were washed twice with 500 μl of 0.5 N NaOH, with overnight incubation at 4°C. The total protein in each chamber was extracted by adding 200 μl of 0.5 N NaOH, with overnight incubation at 4°C. Protein concentration was determined using a Bradford protein assay (triplicate, Bio-Rad). Results are expressed as microgram of protein in each chamber.

Western blot analysis. Proteins were resolved on 4–15% linear gradient Tris/glycine polyacrylamide gels. Equal amounts of mitochondrial protein (5 μg), solubilized in Laemmli buffer (10 mM Tris, pH 6.8, 1.5% SDS, 6% glycerol, 0.05% bromophen blue, 40 mM dithiothreitol), were loaded for SDS-PAGE. Electrophoresis was performed at 150-V constant voltage, and the proteins were then transferred electrophoretically onto Immobilon P membranes (Millipore at 1 mA/cm<sup>2</sup> constant current for 90 min). Blots were blocked for 1 h with 5% nonfat dry milk in wash buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5), rinsed, and probed with rabbit polyclonal anti-cytochrome c (1:2,000 dilution, Clontech), mouse anti-COX IV antibody (1:2,000 dilution, Clontech), mouse anti-Bcl-2 IgG (1:2,000, Zymed), or rabbit polyclonal anti-mouse Bax (1:2,000 dilution, Santa Cruz Biotechnology) overnight at 4°C. The immune complexes were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse goat IgG (1:7,000 dilution, Amersham Pharmacia), using enhanced chemiluminescence (ECL plus, Amersham Pharmacia) with exposure to light-sensitive film (Hyperfilm, Amersham Pharmacia).

Electron microscopy. Cells were fixed on coverslips by immersion in phosphate-buffered 2.5% glutaraldehyde. For light and transmission electron microscopic examination (TEM), the glutaraldehyde-fixed cells were scraped off the coverslips and centrifuged to form a pellet. The pellets were then rinsed in PBS and postfixed for 1 h in phosphate-buffered 1% osmium tetroxide, dehydrated through graded acetones, and embedded in Spurr embedding medium. Semi-thick sections (1–2 μm) were cut, stained with toluidine blue, and examined using an Olympus OM2 light microscope. Thin sections (50–80 mm) were poststained with uranyl acetate and lead citrate and examined using a JEOL 1200EX TEM operating at 60 kV.
RESULTS

Mitochondrial dysfunction evidenced by NADH autofluorescence and membrane potential collapse after osmolality was increased to 700 mosmol/kgH₂O by addition of NaCl. The functional status of mitochondria in mIMCD3 cells was evaluated by measuring NADH autofluorescence and mitochondrial membrane potential with TMRM. As shown in Fig. 1A, supravital staining of mIMCD3 cells growing in control medium (300 mosmol/kgH₂O) revealed punctate intracellular distribution of NADH blue fluorescence that colocalized with the green fluorescence of the mitochondrial-specific fluorophore Mitotracker green. The blue autofluorescence also colocalized with the red fluorescence of TMRM (Fig. 1B). Mitochondrial localization of the blue NADH fluorescent signal was further confirmed by its rapid disappearance after application of FCCP, a mitochondrial uncoupler (not shown).

When the osmolality of the medium was increased to 700 mosmol/kgH₂O by the addition of NaCl, the cells decreased in size and the previously localized NADH signal spread throughout the cell (Fig. 2A). The NADH signal changed in this fashion in numerous (but not all) cells in all experiments (n = 7), but the time course differed among cells, ranging from 45 s to 30 min. In contrast, when osmolality was increased by the addition of NaCl only to 500 mosmol/kgH₂O, the NADH signal did not change in any cells (not shown).

The change in the NADH signal at 700 mosmol/kgH₂O (Fig. 2B, top row) correlated with mitochondrial membrane depolarization, indicated by decreased
TMRM fluorescence (Fig. 2B, bottom row). Although TMRM fluorescence decreased at 700 mosmol/kg H₂O, it did not change significantly at 500 mosmol/kg H₂O (Fig. 2C). Preincubation for 10 min at 500 mosmol/kg H₂O did not prevent the decrease in TMRM fluorescence when osmolality was subsequently raised to 700 mosmol/kg H₂O (Fig. 2C). The mitochondrial membrane depolarization at 700 mosmol/kg H₂O was not reversible. TMRM staining was not restored by returning the cells to the control medium (300 mosmol/kg H₂O).

In contrast to the result with NaCl, addition of urea to a total osmolality of 700 mosmol/kg H₂O (Fig. 3) did not alter NADH fluorescence and did not reduce mitochondrial membrane potential, as analyzed by TMRM fluorescence.

In summary, adding NaCl (but not urea) to a total osmolality of 700 mosmol/kg H₂O causes NADH redistribution out of mitochondria and decreases mitochondrial membrane potential.

**ADP/ATP ratio increases when osmolality is raised to 700 mosmol/kg H₂O by addition of NaCl.** Mitochondrial function was further evaluated by determination of the ADP/ATP ratio. Because ATP synthesis depends on mitochondrial membrane potential, mitochondrial dysfunction produced by high NaCl should cause a rapid increase in the ADP/ATP ratio. Accordingly, the ADP/ATP ratio increased significantly within 1 h after osmolality was increased to 700 mosmol/kg H₂O by the addition of NaCl and remained elevated until at least 6 h (Fig. 4). In contrast, when osmolality was increased only to 500 mosmol/kg H₂O by the addition of NaCl, there was no significant increase in the ADP/ATP ratio.

**Lack of morphological changes in mitochondria after hypertonicity-induced apoptosis.** Mitochondria generally swell during oncotic (necrotic) cell death but either do not change or shrink and display matrix hyperdensity in apoptosis (16). However, in cell culture, mitochondrial swelling has also been observed in apoptosis (13). Mitochondrial swelling can be an important clue concerning the mechanism of apoptosis, because it may cause inner membrane disruption and release of mitochondrial components, including proapoptotic factors. Therefore, we examined mIMCD3 mitochondria by TEM at 300 mosmol/kg H₂O and after 1 and 3 h of hypertonicity (NaCl added to total osmolality of 500 or 700 mosmol/kg H₂O, Fig. 5). No significant alterations in mitochondrial ultrastructure are apparent. Most of the mitochondria exhibit round-to-elongated profiles, with characteristic cristae. Although nuclear chromatin condensation was present in the apoptotic cells at 700 mosmol/kg H₂O at 1 and 3 h, there was no mitochondrial swelling and there were no electron-dense inclusions in the mitochondrial matrix.

**Nuclear condensation and caspase activation produced by high NaCl.** The early mitochondrial dysfunction after the hypertonic stress suggests that mitochondria could have a role in initiating the apoptosis that results. Thus mitochondria can activate at least two apoptotic pathways in response to death stimuli, by releasing apoptosis-inducing factor (AIF), which induces nuclear condensation, or releasing cytochrome c, which activates caspase-9.

We analyzed nuclear condensation by laser scanning cytometry, measuring the brightness of PI staining from each nucleus of mIMCD3 cells growing on chamber slides. Figure 6A shows typical bivariate cytograms plotting the DNA content of single nuclei vs. their brightness. The regions of the plots containing hypercondensed (apoptotic) nuclei are indicated. As shown in Fig. 6B, 4 h after the osmolality was raised to 700 mosmol/kg H₂O by the addition of NaCl, there was a marked increase in the number of hypercondensed nuclei. However, there was no significant change in the number of hypercondensed nuclei after 6 h at 500 mosmol/kg H₂O. There was a steady increase in the proportion of hypercondensed nuclei at 700 (but not at 300 or 500) mosmol/kg H₂O, which was already significant after 1 h.
The rapid nuclear condensation could result from release of AIF from mitochondria and/or the rapid activation of caspases that cause chromatin condensation. To examine these alternatives, we measured caspase-9 and total caspase activation after hypertonic stress. As shown in Fig. 7, caspase-9 activity did not increase significantly after 3 h at 700 mosmol/kg H2O but did increase after 6 h. General caspase activation (Fig. 7), measured as the cleavage of a pan-caspase substrate, increased with a similar time course. At 500 mosmol/kg H2O, there was no significant activation of caspases. Because the nuclear condensation preceded any apparent caspase activation, we investigated additional factors that might play an early role.

Mitochondrial Bcl-2, Bax, cytochrome c, and p53. A decrease in the mitochondrial Bcl-2/Bax ratio is associated with apoptosis. We measured Bcl-2, Bax, COX IV (a protein integral to the mitochondrial inner membrane) by Western blot analysis of mitochondria isolated from cells exposed to high NaCl for 1 or 3 h (Fig. 8A). Hypertonicity dramatically reduced the Bcl-2/Bax ratio 1 and 3 h after osmolality was increased to 700 mosmol/kg H2O by the addition of NaCl. The Bcl-2/Bax ratio decreased less at 500 mosmol/kg H2O (Fig. 8B).

**Fig. 5.** Mitochondrial morphology. Cells were fixed after 3 h of incubation at 300 mosmol/kg H2O (A) or after increase to 500 (B) or 700 (C) mosmol/kg H2O by addition of NaCl. No significant difference in mitochondrial ultrastructure is apparent. Most of the mitochondria (arrowheads) exhibit round-to-elongated profiles, characteristic cristae, and no evidence of electron-dense inclusions in the mitochondrial matrix. N, nucleus. Marker, 200 nM. Magnification, ×45,000.

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addition of NaCl, p53 increases to a higher level than at 500 mosmol/kgH2O, but it is not phosphorylated on Ser-15 as it is at 500 mosmol/kgH2O. Conceivably, the unphosphorylated p53 might translocate to mitochondria, where it could be proapoptotic. Therefore, we measured p53 abundance in the mitochondrial fractions. Only a minor amount of the p53 was detected in mitochondria by Western blot analysis, and no increase with osmolality was observed. Furthermore, by immunofluorescence microscopy (Fig. 8C), there was no apparent localization of p53 to mitochondria at any osmolality.

DISCUSSION

The present studies demonstrate that when the osmolality of media bathing mIMCD3 cells is increased by the addition of NaCl up to 700 mosmol/kgH2O, there is rapid mitochondrial dysfunction, followed by apoptosis. Thus NADH autofluorescence, which is normally localized to mitochondria, becomes dispersed within minutes, consistent with decreased availability of reduced NAD as a substrate for oxidative phosphorylation in the mitochondria. Furthermore, the mitochondrial membranes rapidly and irreversibly depolarize, and the cellular ADP/ATP ratio increases, consistent with rapid inhibition of oxidative phosphorylation. Interestingly, raising the osmolality to the same extent by adding urea does not result in any apparent mitochondrial changes. The mitochondrial changes were the same when osmolality was increased by the addition of NaCl in two steps, first to 500 and then to 700 mosmol/kgH2O, as when the increase to 700 mosmol/kgH2O was made in a single step. There was little if any evidence of mitochondrial dysfunction when osmolality was raised to 500 mosmol/kgH2O by the addition of NaCl. The difference in effects of 500 vs. 700 mosmol/kgH2O is in good agreement with previous reports of a threshold between 650 and 700 mosmol/kgH2O for killing of mIMCD3 cells by apoptosis by high NaCl (35, 45). These correlations suggest that the mitochondrial dysfunction is relevant to the apoptosis.

The mitochondrial dysfunction could be caused directly by changes in matrix volume, secondary to the acute increase in extracellular, and hence cytoplasmic, osmolality. Thus osmotic induction of a mitochondrial matrix volume decrease in vitro adversely affects oxidation of respiratory substrates, the reduction state of cytochromes, and ATP synthesis (26). Hyposmolality has the opposite effect of stimulating mitochondrial function. Hormones that increase mitochondrial activity increase matrix volume, and increased metabolic activity of mitochondria in vitro correlates with the enlargement of the mitochondrial matrix (26). Furthermore, the protein concentration in mitochondria is higher than in the cytosol, probably associated with a higher viscosity that could hinder the movement of metabolic substrates (49). The protein concentration increases when the matrix volume decreases in isolated mitochondria as a consequence of high medium osmolality (46). The result is slowing of diffusion of a
metabolite-sized fluorescent probe (46). Such limited diffusion in a crowded mitochondrial matrix could inhibit mitochondrial function. Adding sucrose or NaCl to the medium decreases mitochondrial matrix volume and reduces mitochondrial respiration and oxidative phosphorylation (17, 18). High KCl has an effect that is similar, but transient. Mitochondrial volume regulation occurs only in the presence of respiratory substrates and is strongly inhibited by uncouplers, indicating that volume recovery is energy dependent (18). Thus when mitochondrial volume is decreased, energy production is compromised, and when cytosolic osmolality increases, increasing the extramitochondrial NaCl concentration, volume regulation apparently can only occur while a change in pH is maintained. The experiments quoted above did not examine effects of hyperosmolality above 400 mosmol/kgH$_2$O, and we are unable to find reports of any that have. Nevertheless, it is tempting to speculate that greater matrix condensation when osmolality is increased to 700 mosmol/kgH$_2$O by the addition of NaCl might irreversibly compromise energy production and trigger apoptosis in mIMCD3 cells. Along this line, ATP depletion of cultured rat kidney proximal tubule cells, induced by hypoxia or chemical inhibitors of mitochondrial respiration, triggers the translocation of cytosolic Bax to mitochondria, cytochrome c release into the cytosol, and apoptosis (44). Drugs and chemicals that affect mitochondrial energy production can also trigger apoptosis in other cell types (4, 14, 38).

We found that raising osmolality to 700 mosmol/kgH$_2$O by the addition of NaCl increases the Bax-to-Bcl-2 ratio in mitochondria. The functions of Bax and Bcl-2 are not completely defined. However, the ratio of...
Bcl-2 ratio may increase the passive nicotinic-induced change in the mitochondrial Bax-to-translocator, to form a channel (12, 33, 42, 48). In view dependent anion channel or the adenine nucleotide mitochondrial membrane proteins, like the voltage-oligomers, a process that could be antagonized by Bcl-2 concentrations of Bax also decrease the stability of planar which are inhibited by Bcl-2 planar lipid bilayers forms pH- and voltage-dependent ion-conducting channels, which are inhibited by Bcl-2 (2). Subnanomolar concentrations of Bax also decrease the stability of planar phospholipid membranes, suggesting direct interaction of the Bax with lipid membranes (7). The pore-forming abilities of Bax may depend on the formation of Bax oligomers, a process that could be antagonized by Bcl-2 (25, 36). Alternatively, Bax could interact with other mitochondrial membrane proteins, like the voltage-dependent anion channel or the adenine nucleotide translocator, to form a channel (12, 33, 42, 48). In view of these observations, we speculate that the hypertonicity-induced change in the mitochondrial Bax-to-Bcl-2 ratio may increase the passive flux of ions and small molecules and cause release of proapoptotic molecules, as well as possibly contributing to the mitochondrial membrane depolarization induced by high NaCl.

The bright autofluorescence of NADH in mitochondria is due to the formation of specific protein-nucleotide complexes in the matrix, which enhances the fluorescence of NADH ~10-fold (5, 6, 20). The hypertonicity-induced decrease in mitochondrial NADH autofluorescence and increase in cytoplasmic autofluorescence could result from movement of NADH and/or the complex-forming proteins from mitochondria to cytoplasm.

With regard to the mechanism of apoptosis, we find that hypertonicity-induced nuclear condensation precedes activation of caspases. A possible explanation is that hypertonicity-induced nuclear condensation is caused by AIF (51) released from mitochondria. AIF is a flavoprotein with NADH oxidase activity that is normally present in the mitochondrial intermembrane space and is released in response to death stimuli (15). Extramitochondrial targeting of AIF, microinjection of recombinant AIF, or addition of AIF to isolated nuclei leads to chromatin condensation (51). Interestingly, AIF can be released from mitochondria without affecting mitochondrial morphology, and studies in AIF null embryonic stem cells indicate that AIF can provide a pathway, separate from caspase activation, required for cytochrome c release in apoptosis during early embryogenesis (28). Another pathway that could be activated by hypertonicity is the release of endonuclease G (endoG) from mitochondria (30, 41). This nuclease is a mitochondrial-specific nuclease that translocates to the nucleus during apoptosis. Once released from mitochondria, endoG cleaves chromatin DNA into nucleosomal fragments independently of caspases. Therefore, endoG represents a caspase-independent apoptotic pathway initiated from the mitochondria (27).

We conclude that our results are consistent with a model in which hypertonicity-induced apoptosis is triggered by receptor-independent mitochondrial dysfunction. The increased mitochondrial Bax/Bcl-2 ratio could trigger the release of AIF and other apoptotic factors from mitochondria. The subsequent nuclear condensation could be a consequence of rapid AIF release, followed by caspase-9 activation. Finally, the apoptosis triggered by high NaCl does not require mitochondrial swelling, favoring the hypothesis that apoptogenic factors are released without rupture of the inner membrane.

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